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(54) Title: CYTOCOHERENT SOLUTE TRANSFER DEVICE

(57) Abstract

The present invention is a method for cytological coherent transfer of cell solutes, including nucleic acids, proteins or carbohydrates from a tissue sample to a microporous transfer membrane. The method comprises selecting a transfer membrane of appropriate composition, pore size and porosity, providing intimate coextensive contact between the sample and transfer membrane, and mobilizing the solute transfer. Mobilization is achieved by thawing a frozen sample on surfactant impregnated membrane and further enhanced by lyophilization, which fixes the sample for storage. The present invention also provides device comprising a microporous transfer membrane affixed to a support comprised of a specimen slide, tissue disc and specimen cassette, each employing transfer membrane selected in accordance with the present invention. The device is adapted for direct and convenient microscopic examination of one or plurality of samples thereon.

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CYTOCOHERENT SOLUTE TRANSFER DEVICE

RELATED APPLICATIONS

This is a continuation-in-part application of copending U.S. Application Serial No. 07/748,558 entitled "Method of Cytocoherent Solute Transfer For Specimen Assay" filed August 22, 1991, by McGrath, which is a continuation-in-part of U.S. Application Serial No. 07/383,446 entitled "Transfer Methods for *in Situ* Assay of Messenger RNA and Proteins" filed July 24, 1989, by McGrath.

FIELD OF THE INVENTION

The present invention relates generally to the cytologically coherent transfer of cell solutes such as nucleic acids, polypeptides/proteins and carbohydrates to microporous substrates and, more particularly, to the preparation of cytocoherent specimens for cytochemical assay for cell solutes.

BACKGROUND OF THE INVENTION

The presence of biochemical markers in patient tissue and fluids can assist in the diagnosis and treatment of a variety of conditions. Assays for such markers are routinely conducted on homogenized tissue samples which are relatively easy to prepare and can be assayed with a high degree of sensitivity. Although advantageous in these respects, the use of tissue homogenates is not without certain drawbacks. For example, assays conducted on homogenized tissue do not accommodate a wide range of cell heterogeneity, leading to possible misinterpretation of assay results and delayed diagnosis. A case in point is breast cancer. While the proliferative tumor cell compartment in breast cancers can vary more than ten times in size, a two-fold change in cell content of epidermal growth factor receptor (EGFR) or estrogen receptor (ER) can result in major changes in cell growth. See Davidson, N. et al., *Mol. Endocrinol.* 1:216-223 (1987); Kawamoto, K. et al., *J. Biol. Chem.* 259:7761-7766 (1984). When receptor content of homogenized tissue is averaged over multiple cell types of various sizes, primary diagnostic alterations in per cell receptor content cannot be differentiated from unrelated differences in cell number or type.

Another drawback in the use of homogenized tissue is that relatively large blocks of tissue are oftentimes required to increase the accuracy of averaged assay results, creating problems in storage and shipment, particularly when the tissue is frozen. An additional disadvantage is that the high degree of sensitivity of such tissue assays is frequently achieved through the use of potentially hazardous radioactive probes which require specialized handling by trained personnel.

An attractive alternative would be the use of "intact" (as opposed to homogenized) tissue sections where biochemical markers are retained in their microanatomically correct location within the cell. Although more accurate in theory, in practice cytochemical assays on such specimens have lacked requisite sensitivity due to loss of cell solutes, including the marker, during specimen preparation and the assay process itself. See Lawrence, J. et al., *Nucl. Acids. Res.* 13:1777-1799 (1985). By cell solute is meant any cell molecule which is or can be placed in solution or suspension for transfer from the cell, including nucleic acids, proteins/polypeptides and carbohydrates. Current methods employed for enhancing detection in cytochemical assays on tissue sections do not compensate for solute loss, but merely amplify the signal, which does not improve the accuracy of quantitative measurements.

Cell solute loss is thought to be attributable to solute diffusion during conventional specimen preparation in which paraffin or frozen thin sections are mounted on nonporous glass or plastic slides and exposed to aqueous media or thawed on the slide. The use of microporous thin film substrates for transfer has been explored, but not satisfactorily applied to the preparation of cytocoherent specimens for cytochemical assay. For example, microporous membranes have been used as cell culture substrates and for bacterial colony transfer and assay. Gabriels, J. et al., *Biotech.* 5:731-732 (1987); Grunstein, M. et al., *PNAS (USA)* 72:3961-3965 (1975). Other methods directed to preparing cell specimens have not recognized or employed the materials and conditions necessary for cytologically coherent solute transfer with accurate planar definition with respect to cell organelles. Seshi, *Anal. Biochem.* 157:331-342 (1986); Kendall, *Meth. Enzymol.* 168:327-338 (1989); Cassab, G.I. et al., *Cell Biol. Int. Reports* 13:147-152 (1989); Pont-Lezica, R.F. et al., *Anal. Biochem.* 182:334-337 (1989); Kendall, M.E. et al., *Endocrin.* 121:2260-2262 (1987); Cassab, G.I., et al., *Cell Biol.* 105:2581-2588 (1987); Platt, S.G. et al., *Anal. Biochem.* 162:529-535 (1987). By cytologically coherent or cytocoherent transfer is meant transfer which preserves the location of the target cell solute with accurate planar definition relative to cell microanatomy and organelles with which the solute associates. By planar definition is meant the two-dimensional orientation of the solute with respect to the specimen image, i.e. cell and organelle anatomy, at the level of microscopic resolution of the biological membranes which comprise and delimit the cell and its organelles.

A further problem in current cytochemical technology involves sample preservation and storage. The most common forms of sample preservation are immersion fixation and paraffin or resin embedding. However, enzymatic and immuno-

histochemical studies have clearly shown these methods to have adverse affects on bioactivity. Herskovits, T. et al., *Fed. Proc.* 27:771-776 (1968); Herskovits, T. et al., *Science* 163:282-284 (1969); Tanford, C. et al., *J. Biol. Chem.* 237:1168-1175 (1962); Singer, S.J., *Adv. Protein Chem.* 17:1-33 (1962); Sjostrand, F.S., *J. Ulstruct. Res.* 55:271-275 (1976); Kauzmann, W., *Adv. Protein Chem.* 14:1-39 (1959); Kellenberger, E., *Trends Biochem. Sci.* 3:135-145 (1978) and Sternberger, L.A., "Immunocytochemistry" 2nd ed. (John Wiley & Sons, NY (1979)). Another approach, "quench freezing" at ultra-low temperatures also has certain drawbacks. Although greater bioactivity is observed, the clinical utility of this method is severely limited due to problems associated with low temperature storage (-70°C or below). Retrieval, shipment and expense as well as destruction of the specimens due to ice recrystallization and devitrification are among the problems encountered with this method.

An alternative preservation method is freeze-substitution in which specimens are quench frozen in melting solvent in liquid nitrogen and rapidly transferred to partly frozen ice-solvent for ice displacement. The specimens are then infiltrated with high cross-linking polymer, such as glycol methacrylate, under vacuum and polymerized at low temperatures. This method is attractive because the embedded (*i.e.* permeated with polymer) specimens can be stored long-term at room temperature. However, embedding with methacrylate creates stearic barriers to target binding below the embedded surface and encourages target denaturation. Sjostrand, F.S., *supra*, Kauzmann, W., *supra* and Roth J., et al., *J. Histochem. Cytochem.* 38:95-101 (1990). Target diffusion and antigen instability are also likely in freeze-substituted specimens.

Another problem encountered in quench freezing and storage below -70°C is channeling in the sample. Channeling, which results from ice crystal formation and sublimation, causes undesirable desiccation and deterioration of tissue when this method is employed. Attempts at limiting channeling include distillation drying which is drying at temperatures below -90°C, and drying with alcohol or acetone at ultra-low temperatures (freeze-substitution method).

It would thus be desirable to provide a method for the transfer of cell solutes to a substrate in which cell microanatomy and solute location with respect to cell structure is preserved. It would also be desirable to provide a transfer method in which target solute retention during transfer in a cytologically coherent configuration is enhanced or optimized. It would further be desirable to provide a method for preparing specimens for assay with accurate planar definition with respect to cell organelles and

high visual resolution thereof. It would also be desirable to reduce or minimize lateral diffusion during transfer and specimen preparation in order to maintain high microscopic resolution. It would also be desirable to provide alternatives to or reduce the dependence on radioactive probes in cytochemical assays, yet maintain sufficient signal intensity. It would further be desirable to provide a specimen preparation method which results in cell or tissue specimens which can be easily stored and shipped at ambient temperatures. It would also be desirable to provide a method for preserving tissue for cytochemical assays which allows for long-term storage without negatively impacting bioactivity. It would further be desirable to provide a method of fixation which increases target solute mobilization. It would also be desirable that the method be applicable to both tissue and fluid samples of either animal, microbial or plant origin. It would further be desirable to provide a method for preparing specimens for cytochemical assay which is easy and quick to conduct, without sacrificing accuracy of the results. It would be also desirable to provide devices suited for the practice of the present invention and specifically adapted for direct and convenient microscopic examination.

SUMMARY OF THE INVENTION

The present invention provides a method for transferring cell solutes to a microporous substrate which decreases target cell solute loss while maintaining cytological coherency of solute transfer. The present invention further provides a method of preparing a cytocoherent specimen for assay for a target cell solute generally comprising the steps of:

a) providing a tissue or fluid sample;

b) providing a microporous transfer membrane, the step of providing the transfer membrane further comprising the step of selecting a membrane with the following characteristics:

1) pore size no greater than approximately 0.1 µm;

2) porosity which does not disrupt substantial molecular continuity between the sample and transfer membrane when they are contacted; and

3) a composition with binding affinity for and having sufficient binding capacity for the target solute and soluble contents of the specimen which are selected for transfer and which is resistant to the agents used in the transfer method;

c) providing substantially molecularly continuous contact between the sample and the transfer membrane under conditions favorable for target solute

transfer and unfavorable for lateral diffusion of solute on the transfer membrane; and

d) mobilization of the target cell solute.

Absent microscopic resolution constraints, the method of the invention also comprises a method of transfer wherein step (b)(1) comprises providing a microporous transfer membrane with a pore size no greater than the average thickness of the membrane domain of the biological membrane with which the target solute associates.

The present invention further comprises a method for preparing a specimen for assay for the presence of a target solute generally comprising the steps of (a)-(d), further comprising the step of providing means for detecting or assaying for the target solute on the transfer membrane. Cytochemical assays performed in accordance with the principles of the present invention include immunological, enzymatic, *in situ* hybridization and *in situ* PCR assays on target solute immobilized on the transfer membrane. The transfer membrane is then also selected for its resistance to reagents used during assay and for a low degree of nonspecific binding. Porosity of the transfer membrane is also selected to enhance signal intensity. The method of the present invention further comprises visualizing assay results with respect to cell microanatomy, which can include the steps of staining the cell organelles and clearing the transfer membrane for microscopic examination.

A further object of the method of the present invention utilizes lyophilization to fix tissue on microporous transfer membrane through dehydration under vacuum at low temperatures. Lyophilization also enhances mobilization of target solutes through the process of ice crystal formation, reformation and sublimination, *i.e.* channeling, which creates new reaction surfaces within tissue and normalizes density differences that naturally occur in tissue. In contrast to freeze substitution and distillation drying methods which attempt to eliminate or minimize channeling, the drying step of the present invention is manipulated to optimize the level of channeling to create channels as large and diffuse as possible without distorting cell microanatomy. Gersh, I., et al. "In: Biological Applications of Freezing and Dying" (R.J.C. Harris, ed.) Academic Press (NY 1972) pp 337-384.

In order to practice and observe the results of the cytocoherent transfer process of the present invention, a variety of devices for carrying out the process of the invention and associated holders for positioning the tissue specimens on the microscope stage of a light microscope are provided. The device of the invention generally comprises a transfer membrane selected in accordance with the principles

of the present invention, preferably affixed to an underlying support. One embodiment of the device includes a conventional microscope slide or coverslip with a transfer membrane of the present invention affixed to the surface of either the slide or coverslip by any suitable method such as casting, use of adhesives or welding. Another embodiment of the device comprises a tissue disc including a disc-shaped support member and a plurality of tissue sites circumferentially positioned around the support member where each tissue site includes the transfer membrane of the present invention. A tissue disc holder positions the tissue disc relative to the microscope such that each tissue site can be microscopically viewed. A third embodiment of the device includes a continuous strip of support material having spaced apart tissue sites, where each tissue site includes a transfer membrane. The continuous strip is wound in an unused cassette, such that in use, the unused cassette is attached to the microscope in association with a used cassette such that the continuous strip is threaded from the unused cassette to the used cassette across the microscope stage for viewing each individual tissue site.

Other features and advantages of the present invention will be become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 (A-C) are light micrographs (2X magnification) illustrating the appearance of thin breast tissue sections (5μm) on nitrocellulose membrane (0.05μm pore size): (A) after thaw-mounting and air-drying tissue (encircled); (B) after immunocytochemical staining; and (C) after counterstaining and clearing membranes. The finished specimen was placed atop lettering.

Figures 2(A-F) are light micrographs of H&E stained thin (5μm) breast specimens thaw-mounted on nitrocellulose membranes (0.05μm pore size) illustrating the mrophologic integrity of biologial specimens on microporous membranes suitable for diagnosis: (A) malignant cells of ductal carcinoma (NOS) (X400), illustrating mitotic figure (arrow) and nuclear pleiomorphism; (B) benign papilloma (X250); (C) benign intraductal hyperplasia (X250); (D) intraductal carcinoma, comedo type (X250); (E) cellular detail of benign papilloma in (B) (X350); and (F) solid type intraductal carcinoma with areas of microinvasion (X250).

Figures 3(A-D) are light micrographs illustrating that Epithelial Membrane Antigen (EMA) and Her-2 receptor proteins can be resolved in a cytocoherent manner on nitrocellulose membranes (0.05μm pore size): (A) EMA-specific immunochemical

staining reaction in secretory lobules (X150); (B) EMA-specific reaction in non-secretory terminal ducts and interspersed invasive cancer cells; (C) Her-2 cell membrane-specific reaction in a clinging-type intraductal carcinoma (X200); and (D) Her-2-specific reaction in a comedo-type intraductal carcinoma (X440).

Figures 4(A-D) are light micrographs (X350) illustrating the effect of membrane pore size and membrane pre-wetting on Her-2 signal intensity and resolution; tissue sections were thaw-mounted and immunoassayed on nitrocellulose membranes: (A) 0.05μm pore size, tissue mounted on dry membrane, air-dried and fixed; (B) 0.05μm pore size, tissue mounted on membrane pre-wet with PBS, air-dried and fixed as above; (C) 0.45μm membrane, tissue mounted on dry membrane, air-dried and fixed as above; and (D) 0.45μm pore size, tissue mounted on pre-wet membrane air-dried and fixed as above. Insets are copy-stand photographs (X2) of the correspondingly treated tissue sections.

Figures 5(A-D) are graphs illustrating the substantially greater sensitivity of Her-2 immunocytochemical assays on tissue sections affixed to transfer membranes compared to those affixed to glass slides.

Figure 6(A-C) are light micrographs photographically illustrating the greater sensitivity of Her-2 immunocytochemical assays on breast cancer tissue thaw-mounted on nitrocellulose membranes (0.05μm pore size) than on silanized glass slides: (A) tissue thaw-mounted on nitrocellulose and reacted with Her-2 (mAb-1) (B) tissue thaw-mounted on silanized glass slides and reacted with Her-2(mAb-1) (C) tissue thaw-mounted on nitrocellulose and reacted with EGFR (Ab-4).

Figure 7 are light micrographs (X 350) illustrating the effect of membrane pore size on cytomorphology of thin sections of one breast carcinoma thaw-mounted on dry nitrocellulose membranes, air dried, fixed with paraformaldehyde, and counterstained with hematoxylin and eosin: (A) 0.05μm pore size; and (B) 0.45μm pore size.

Figure 8 is a schematic representation showing the effect of pore size on planar definition to target solutes: (A) 0.05μm pore size; and (B) 0.45μm pore size.

Figure 9 illustrates specific cytoplasmic localization of Her-2 mRNA in breast cancer cells of three different tissues affixed to nitrocellulose transfer membranes (0.05μm pore size): (A) normal cells in hyperplastic duct (X350); (B) comedo-type carcinoma *in situ* (X200); and (C) infiltrating ductal carcinoma (X200).

Figures 10(A) and 10(B) illustrate a specimen slide device and a coverslip device of the present invention.

Figure 11 illustrates a tissue disc comprised of a plurality of tissue sites including a transfer membrane of the present invention.

Figure 12 illustrates a tissue disc holder for positioning the tissue disc of Figure 11 in relation to a microscope.

Figure 13 illustrates a lyofile container for filing and storing the tissue disc of Figure 11.

Figure 14 illustrates a cassette device comprising a continuous strip of material including individual tissue sites having transfer membranes in which an unused cassette and a used cassette position the strip relative to a microscope.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

A. OVERVIEW

The method of the invention generally comprises the steps of preparing a sample, selecting an appropriate microporous transfer membrane, and intimately contacting the sample and the membrane and mobilizing the target solute under conditions favoring transfer and discouraging lateral diffusion of the solute on the transfer membrane. The sample can comprise any fluid or tissue containing or to be assayed for the presence of a cell solute of interest, i.e. target solute. It will thus be appreciated that the method of the invention contemplates not only the transfer of target solutes, but the preparation of specimens for assay for target solute which may or may not be present in the sample.

Once the sample is prepared, it is intimately contacted with the microporous transfer membrane. By intimate contact is meant contact which is substantially continuous at a molecular level. Intimate contact is facilitated by adequate surface smoothness of the transfer membrane, determined by membrane characteristics such as pore size (as measured by pore diameter) and membrane porosity. For solute transfer to be cytocoherent with respect to cell structure, membrane contact must extend substantially over the entire diameter of the cell, hereinafter referred to as coextensive contact. Intimate coextensive contact can be accomplished by fractionating cells prior to their affixation to the membrane or, in the case of whole cells, applying positive or negative pressure to flatten and affix the cells to the membrane.

To facilitate transfer of the target solute to the microporous membrane, the soluble contents of the sample are mobilized. By mobilization is meant providing physical mobility of the solute, without destroying the membranous cell matrix. Mobilization is generally achieved through devitalization of the cell sample, i.e. cell death, but is

preferably increased through physical and chemical means resulting in poration and the disruption of chemical bonds. Surfactants, described in more detail below, are especially effective in facilitating mobilization. Lyophilization to fix the sample, further described below, can also be used to enhance mobilization through the formation of channels in the sample.

In accordance with the present invention, the characteristics of the microporous transfer membrane, including chemical composition, pore size and porosity are selected to achieve cytologically coherent solute transfer. Membrane composition is selected to provide binding affinity for and sufficient binding capacity for any solutes desired to be transferred to the membrane. Membrane composition should also be selected for its resistance to any reagents used during transfer and assay. The membrane and transfer conditions, such as, *e.g.* membrane dryness at the time of initiation of transfer, are further selected to minimize or reduce lateral diffusion during transfer.

Pore size of the transfer membrane is selected to result in accurate planar definition of solute relative to the cell and cell organelles, hereinafter collectively referred to as cell structures, as defined by their membrane dimensions. Suitable pore size for adequate planar definition in accordance with the practice of the invention is determined by two factors: first, the smallest unit of cytological resolution desired, and, second, microscopic resolution constraints.

In the practice of the present invention, the unit of cytologic resolution desired is the biological membrane which defines cell microanatomy. Using simply this criterion, accurate planar definition in accordance with the present invention would be achieved by selecting a pore size no greater than the approximate thickness of the biological membrane of the cell structure with which the target solute is associated. It will be appreciated that the cell structure with which the target solute associates can be either a specific cell organelle, in which case the biological membrane is the organellar membrane, or the cell generally, in which case the membrane is the cell or plasma membrane.

It is well know by those skilled in the art that the delimiting and compositional membranes of animal, plant and microbial cells and organelles (hereinafter collectively referred to as cellular or biological membranes) exhibit substantial uniformity, with the membrane lipid bilayer and integral proteins classically considered to be approximately 0.01μm thick. Absent microscopic constraints, cytologic preparation effects and membrane variability, theoretically "perfect" planar definition would thus be acheived

using a pore size no greater than approximately 0.01μm in diameter. However, cytologic preparation of the specimen in accordance with the invention, including the adsorption of peripheral proteins, the use of fixatives, the degree of dehydration and other factors affect membrane dimensions and can result in a prepared biological membrane with adsorbed peripheral proteins (hereinafter referred to as the biological membrane domain) ranging from about 0.05μm to about 0.1μm in thickness. Thus transfer membranes of this higher pore size may also be suitable.

Microscopic resolution constraints also come into play in determining suitable pore size of the transfer membrane. Given the fact that the smallest unit of resolution of the light microscope generally used in cytopathology is approximately 0.1μm, when a light microscope is used in the practice of the invention, transfer membranes of such pore size are suitable even should the biological membrane domain be slightly less in thickness, since diffusion to those limits would not be detected. However, in other applications utilizing instruments with better resolving power (i.e. lesser value), *e.g.* electron microscopes, the pore size of the transfer membrane should be correspondingly decreased to at least the average thickness of the biological membrane domain.

When an asay is to be conducted, porosity of the transfer membrane is also selected to enhance signal intensity. For example, numerous small pores generally enhance signal intensity. As described above, membrane pore size and porosity are also selected to provide a smooth surface for intimate contact with the sample.

Once the specimen is prepared, cytochemical assay for a given solute or set of solutes can be performed on the specimen. Cytochemical assays can be immunological, enzymatic or hybridizational in nature. Specimens prepared in accordance with the method of the invention are ideal for *in situ* hybridization assays which utilize nucleic acid probes for hybridization with the target solute. Further visualization of assay results can be accomplished in a variety of ways, including staining the cell organelles, then chemically clearing the transfer membrane to transparency. Cell microanatomy and localization of the target solute or set of solutes can then be determined with the aid of a microscope and/or image analyzer.

The method of the present invention provides a spatially patent image of the location and relative quantity of specific nucleic acids, proteins, carbohydrates or other cell solutes relative to subcellular structure. Microanatomical structure is preserved with accurate planar definition of solute relative to cell and organelle anatomy. By utilizing the method of the present invention, cell solutes, notably mRNA and proteins,

normally lost by washing away using conventional technology are retained *in situ* in the transfer membrane. Lateral diffusion of solute molecules during transfer in accordance with the method of the invention is insignificant with respect to the microscopic resolution of specific cell organelles. Assay results on specimens prepared in accordance with the method of the present invention are thus more accurate and reproducible. Although it will be appreciated that radioactive probes can be utilized with the method of the invention, their use can be avoided if desired because the enhanced sensitivity of the assay of the invention permits use of assays relying on *e.g.* color, luminescence and fluorescence, previously considered of lower sensitivity. Staining (or counterstaining) techniques known to those skilled in the art can be further employed for visualization of cell microanatomy and assay results.

Specimens prepared by the methods of the invention can also be stored and shipped at room temperature, thereby simplifying storage and the logistical aspects of interlaboratory transfer of frozen cell samples specifically for cytochemical assay. Specimens fixed by lyophilization, described in more detail below, are particularly useful in this respect. The method of the invention requires a minimal number of manipulative steps and may be employed with extremely small samples of cells, even single cells, thus expanding diagnostic tests to previously refractory samples.

B. DETAILED DESCRIPTION

Sample Preparation and Transfer Membrane Contact

The sample can be of any origin, animal, plant or microbial, and can be obtained directly from the organism or from cell culture. The sample can comprise solid tissue or biological fluids such as blood, plasma, effusions, urine, saliva, plant fluids and the like. The sample can also be of any shape, mass or cell number, and can comprise whole or fractional cells. However, the sample is prepared and contacted with the transfer membrane in such a way that there will be substantially continuous microscopic contact between the sample and membrane with minimum overlap between any cells or cell matrices transferred to the membrane. Intimate contact should also be coextensive with substantially the entire diameter of the cells introduced to the membrane. Coextensive contact can be accomplished by the use of tissue sections in which cells are fractionated or, in the case of whole cells, the application of vacuum or pressure to the cells on the membrane. It will be appreciated however, that although a specimen of the invention is prepared under conditions providing intimate coextensive contact of cells therein, the sample may not actually contain cells.

For example, a fluid sample such as urine, which normally does not contain cells, may be screened for the presence of cells, as well as specific target solutes.

With solid tissues, intimate coextensive contact with minimal overlap can be achieved by placing a thin tissue section preferably less than one cell diameter, (*e.g.* about 3-8μm for nucleated cells) prepared by means of a cryostat or microtome, flat on the transfer membrane. Thin tissue sections are electrostatically attracted to transfer membranes currently in use, such as nitrocellulose, polyvinylidine difluoride (PVDF) or nylon, and affix smoothly and continuously to the microporous membrane without physical encouragement. Unlike non-porous glass or plastic which require special pre-coating with poly L-lysine or 3-aminopropyltriethoxysilane (or comparable material) for tissue to adhere well (Rentrop, M. et.al. *Histochem. J.* 18:271-276 (1986)), once dried onto the surface of the aforementioned membranes, tissue sections cannot be removed intact. Even vacuum or pressure washing or washing under high stringency conditions used for *in situ* hybridization (*e.g.* 6X SSC, 0.5% SDS, 80°C for 2 hours) will not dislodge tissue sections once dried.

Intimate coextensive contact and affixation to the transfer membrane can likewise be accomplished by centrifuging a dilute cell sample onto the surface of the membrane. The Cytospin apparatus manufactured by Shandon, Inc. is convenient for this purpose. This alternate method is especially useful with fluids such as whole blood or plasma, effusions, urine, saliva and the like. Vacuum suction can also be used to pull cells from dilute suspensions onto the membrane. Appropriate contact can also be accomplished by making a tissue imprint on the surface of the membrane. See eg. Cassab, G.E. et al., *Cell Biol. Int. Reports* 13:147-152 (1989); Pont-Lezica, R.F. et al., *Anal. Biochem.* 182:334-337 (1989). Generally, a cut is made into a block of fresh tissue to fractionate and expose cells, and the cut surface is pressed to the membrane surface. When the tissue block is removed, a cellular imprint of the cut surface remains on the membrane. It will be appreciated that the method of the present invention requires that the sample be prepared and handled so that contact between cell or cell matrix (in the case of thin sections less than one cell thick) and the microporous membrane is substantially microscopically continuous.

Transfer Membrane Preparation and Selection

(1) Membrane Preparation

For convenience of handling, the transfer membrane can be cut to an appropriate size and affixed to a glass or plastic microscope slide with adhesive, by welding or through other means such as casting or coating. For some applications, the

membrane may be stretched across an opening in a solid frame (with the tissue in the opening) for pressure or vacuum washing of the sample.

The transfer membrane can be pretreated with wetting agents to reduce interfacial tension below critical water entry levels for that specific pore size and shape and allow the small volume of cell-bound water and mobile solute in the sample to penetrate the membrane locally via capillarity. The advantage of local wetting on dominantly hydrophobic membranes such as nitrocellulose is that the solute would also be spatially well-confined. However, lowering the interfacial tension more generally, for example, by placing the tissue on water-soaked membrane can overcome the localizing effect of hydrophobicity and allow the solute to laterally diffuse in a superficial water plane, resulting in a diffuse signal. Thus, solute transfer on hydrophobic membranes is preferably conducted on non-prewetted membranes, i.e. dry at the initiation of transfer, to minimize lateral diffusion.

(2) Membrane Selection

The general properties of the microporous transfer membrane selected in accordance with the method of the present invention include membrane composition, pore size, porosity and surface smoothness. It will be appreciated that although all membrane characteristics, such as binding capacity, pore size and porosity can be individually optimized, each characteristic is selected in light of the others to provide a membrane which overall is suitable for cytocoherent transfer in accordance with the principles of the present invention.

(a) Membrane Composition

Microporous materials commonly used for chromatographic transfer of nucleic acid or protein from gels are suitable for the practice of the present invention. Examples of these materials include, but are not limited to, nitrocellulose, PVDF and nylon or composites thereof. All have sufficient tensile strength and clear well enough in histologic mounting media for use in the method of the invention. However, material-related performance differences (*e.g.* in surface tension, ease of blocking, and resistance to alcoholic, acetone or formaldehyde fixatives and the like) may affect choice of the membrane material to suit specific needs which can be determined by those of ordinary skill in the art. It is, however, necessary that the material composition of the membrane be resistant to the agents used in the transfer method of the invention, and also resistant to those used in any cytochemical assays conducted on the sample. The material of the membrane also preferably exhibits optical clarity in histological mounting media.

The composition of the membrane must also have binding affinity for all cell solutes of interest and sufficient binding capacity for the solutes to be transferred from the cells in contact with the membrane. For example, an epithelial cell contains, on average, approximately 12ng soluble protein and 0.4ng RNA, in an area of approximately 300μm (unpublished data of inventor). Nitrocellulose, PVDF and charged nylon all have capacities sufficient to bind all the soluble protein and RNA contained in each cell.

(b) Transfer Membrane Pore Size

Transfer membrane pore size must be selected to meet the following criteria: surface smoothness sufficient to allow intimate contact with the sample and accurate planar definition of solute relative to cell microstructure after transfer. Generally speaking, smaller pore size increases both relative surface smoothness and planar definition.

To achieve satisfactory planar definition in accordance with the present invention, absent microscopic resolution constraints, pore size should be no greater than the average thickness of the biological membrane or membrane domain of the cell structure with which the target solute is associated. Absent both microscopic resolution constraints and cytologic preparation and adsorbed peripheral proteins effects, and given the substantial uniformity of biological membranes, pore size of no greater than about 0.01 μm would ensure accurate planar definition of the solute relative to cellular structure. However, as discussed above, in the practice of a preferred embodiment of the present invention using prepared specimens and the light microscopic pore size no greater than about 0.1 μm will generally be suitable for such applications. This pore size will also provide a sufficiently smooth surface for intimate contact with the sample. Due to the currently availability of 0.05μm pore size transfer membranes, membranes of such pore size are especially convenient for the practice of the present invention.

The degree of planar definition observed in the transfer of Her-2 receptors from cell membrane to nitrocellulose transfer membranes (Figure 4A-D) can be predicted from the Figure 8 schematic illustrating the size relationships involved. As shown in Figure 8A, the use of transfer membranes with uniformly spaced pores 0.05μm in diameter will confine target solutes transferred from affixed cell and organelle membranes to a distance of only 0.05μm from the outer boundary of the membrane domain, a distance well within the resolving limits of conventional light microscopes. The use of transfer membranes with 0.45μm pores, as shown schematically in Figure

8B, results in lateral solute diffusion to a distance greater than resolving power and predictably blurred images.

(c) Membrane Porosity

Transfer membrane porosity is selected, as is pore size, to provide a sufficiently smooth surface for intimate contact between the sample and microporous membrane. Porosity is defined herein as the pore number per membrane surface area. Generally speaking, an increase in porosity will decrease surface smoothness. It will be appreciated, however, that pore size will also interact with pore number to impact on surface smoothness.

When cytochemical assays are to be performed on the specimen, porosity is further selected to enhance signal intensity. Generally, more numerous small pores will enhance the signal. However, porosity should not be so great as to unduly sacrifice membrane strength.

(d) Membrane Surface Smoothness

As described above, the surface of the microporous transfer membrane must be sufficiently smooth to allow intimate contact between the sample and the membrane. Surface smoothness is, as noted above, primarily a function of pore size, i.e., smaller pore size generally resulting in a smoother surface, and porosity, i.e., less pores resulting in smoother surface.

Mobilization of Solutes for Transfer

The integral contact made between cells or the cut surfaces of cells and the microporous transfer membrane provides the necessary physical access for target solute transfer. Although mobilization of cell solutes occurs by virtue of devitalization, i.e. cell death, mobilization of solute for transfer is preferably facilitated by the poration and/or the disruption of chemical bonds in the cell membrane and, in plants, the plant wall by the following additional methods:

(1) Freeze-thawing

Freeze-thawing cells or cell matrices on the microporous transfer membrane surface mobilizes cell-bound solutes via physical poration of organellar membranes and disruption of chemical bonds (*e.g.* denaturation of proteins, dissociation of nucleoproteins and the like). To be effective, freezing should be rapid to minimize ice crystal formation and incumbent morphological distortion. A preferred method is to dip tissue into liquid nitrogen and store at -80°C. Thawing must also be rapid to minimize morphological distortion, but a thin or single layer of cells or fractional cells on membranes can be thawed conveniently at room temperature.

Mobilization can be further enhanced through lyophilization of the sample. As described in more detail below, lyophilization is a fixation method which is believed to also increase mobilization through the process of ice crystal formation and sublimination which creates new reaction surfaces and normalizes density differences in biological tissue. As previously described, channeling causes desiccation and deterioration in tissue samples when samples have been lyophilized directly on non-porous supports. However, when tissue sections are lyophilized on a microporous transfer membrane, the result is enhanced mobilization of target solute for transfer into and retention by the membrane, as well as normalization of regional density differences in the section of tissue which normalizes access to target by reactants. Ice recrystallization rates at the vacuum sublimation step are manipulated to optimize channeling by making channels as large and diffuse as possible without distorting cell microanatomy at 400X.

(2) Chemical treatment

Cell-bound solutes can likewise be mobilized for transfer by chemically treating cells or cell matrices. Low molecular weight alcohols particularly methanol and ethanol, are effective at about 70% (V/V) concentrations or greater. Formaldehyde-based reagents (about 1-10% by volume) are also effective mobilizers because they create pores in cell membranes and disrupt inter- and intra-molecular bonds. Surfactants are especially effective mobilizers because they not only create pores in cell membranes and disrupt both hydrophobic and hydrophilic interactions that bind molecules, they also reduce surface tension of hydrophobic membranes and precipitate high molecular weight solutes into membrane matrices. Surfactants also help facilitate long-term storage of frozen specimens because they accentuate the effect of film surface pores to facilitate bonding of thaw-mounted tissue sections to the film in a tight and continuous way and they are also antimicrobial. Particularly suitable surfactants are those used in the isolation of intact cell membranes and those used to isolate antigenically active proteins from cell membranes. Examples include but are not limited to the anionic surfactants sodium dodecyl sulfate (SDS) and sodium deoxycholate salt (DOC) and the non-ionic surfactants polyvinylpyrrolidone (PVP) and glucopyranosides.

Surfactants can be applied to the microporous membrane or film by soaking the dry film in a dilute solution or by spraying a dilute solution onto the dry film and then drying the surfactant solution in the film. The terms "membrane" and "film" are sometimes used interchangeably herein which will be evident from the context in which

the term is used. Surfactants can also be added to the film material by mixing a dry powder into the casting solution such as generally described in U.S. Patent No. 5,073,344 issued to Smith et al. Such treatments are designed to distribute surfactant uniformly in and on the film. It is also desirable in some applications of the method of the present invention to restrict the surfactant to the film surface to foster the effectiveness of the surfactant but to minimize the consonant negative effects of surfactant within the film to weaken binding of target to film material and facilitate washing of bound targets out of the film material during cytochemical assay. For this application of surfactant, the void volume of the film can be filled with a non-miscible fluid to which the film material is also resistant (*e.g.* butanol in the case of nitrocellulose) before the addition of a dilute surfactant solution to the surface of the film. The surfactant is then dried on the film surface as described above. A thin film of surfactant-loaded film material can also be cast onto the surface of a second, surfactant-free thin film material to achieve the desired effect.

(3) Other methods

Other methods of mobilization contemplated within the scope of the present invention include but are not limited to electroporation or sonication.

Mechanism of Transfer of Solute to Membrane

Transfer of mobilized solutes from cells to microporous transfer membranes occurs when a critical entry pressure is exceeded, with solutes moving into the membrane by capillarity. Immobilization on hydrophobic membranes such as nitrocellulose, nylon, PVDF and the like can occur by several mechanisms, notably by excluding water and forming hydrophobic (van der Waals) and/or electrostatic interactions and salt bridges.

Preservation of Cellular Microstructure

Cell matrices are preferably preserved or fixed on the membrane so that the specimen can be stored at room temperature without solute movement or adverse effects on organellar morphology. Any conventional cell fixative will be satisfactory. In practice, mobilization of solute for transfer and fixation steps can also be accomplished concordantly using alcohols or formaldehyde-based fixatives. Lyophilization, described in more detail below, is a preferred method of the present invention and does not require chemical solvents to foster mobilization.

(1) Lyophilization

Specimens are preferably fixed by lyophilization. Since in lyophilization there is no liquid phase, target diffusion is practically eliminated. Lyophilization also eliminates the denaturation problems inherent in chemical fixation and embedding fixation

methods. Lyophilization is well-suited for long-term storage because air (O_2) as well as water is removed from tissue. There is also no need for storage at ultra-low temperatures, and preservation and associated problems are thus eliminated. Practically, lyophilization is fast, easily incorporated into an automated system and the necessary equipment is simple, inexpensive and can be found in most pathology laboratories. Furthermore, as described above, lyophilization is believed to facilitate mobilization of target solute through channeling.

Cytochemical Assay

After transfer and fixation, assays can be performed to identify, quantitate or localize a given solute or set of solutes that has been transferred. Prior to assay or detection of a specific solute molecule, the target solute can be modified (*e.g.* amplified by polymerase chain reaction) to enhance signal intensity. The specimens prepared in accordance with the method of the present invention are ideal for immunological and *in situ* hybridization assays in which antibody or nucleic acid probes are used to detect target proteins and nucleic acids. See *e.g.*, Angerer, L.M. et al., *Meth. Enzymol.* 152:649-661 (1987). It will be appreciated that these or other assays conducted in accordance with the present invention can utilize radioactive probes. However, assays relying on colorometric changes, luminescence and fluorescence, followed by visualization of cell microanatomy, are preferred.

Although immunological and *in situ* hybridization assays are easily performed on specimens prepared in accordance with the present invention, it will be appreciated that any assay protocol conventionally used in cytochemistry can be employed as long as it is compatible with transfer membrane chemistry.

Visualization of Assay Results

After assays have been completed, the assay results are preferably also visualized with respect to cell microanatomy. For example cells or cell matrices can be stained (or counterstained) to reveal their cellular and subcellular structure. This can be accomplished by any conventional cytochemical methods and reagents that are compatible with transfer membrane chemistry. These include for example, staining with hematoxylin, eosin or nuclear fast red.

After staining cells and/or cell matrices, membranes can be cleared or transparentized for transmitted light microscopy. Clearing is done simply by adding an inert fluid with a refractive index that matches that of the membrane material. For example, the refractive index of nitrocellulose is 1.50, that of PVDF is 1.42. Immersion oils or other clearing agents such as xylene or terpene-based products such as

Permount are commercially available with these and other indices of refraction. If desired, a permanent specimen can be prepared by mounting a coverslip onto the membrane (mounted on a microscope slide) by means of plastic or gum-based media selected to match the refractive index of the membrane.

Alternatively, visualization can also be accomplished by metabolic incorporation of markers by the cells prior to specimen preparation. For example, labelling viable cells with radioactive precursors of macromolecular targets.

Devices

In order to observe the results from the above-described method of cytocoherent transfer, the tissue specimens are generally viewed with a conventional light microscope. As discussed below, devices used in the practice of the method of the invention are adapted to be secured to a microscope stage of the microscope. A variety of configurations for performing this task are within the scope of the present invention.

Devices for the practice of the present invention generally comprise a laminate of at least two layers: a transfer membrane layer and an underlying support layer. As discussed above, the transfer membrane layer comprises a porous polymeric material specially constructed to meet the morphologic and solute binding requirements of the cytocoherent transfer method. The key characteristics of the transfer membrane include having a refractive index enabling the transfer membrane to be translucent during microscopic viewing, and that the transfer membrane should have a binding capacity which will bind the majority of the solutes in the tissue sample. Examples of suitable transfer membranes include nitrocellulose, derivatized nylon such as Nytran® and PVDF-based materials such as Immobilon P. Additionally, the transfer membrane should have a minimum average thickness of about 0.5 mils for sample sizes appropriate to practice the present invention in order to accommodate the entire volume of water held by the sample and to facilitate washing of the sample in order to reduce impurities. However, greater thicknesses on the order of 5.0 mils may be applicable as determined by optical viewing, washing efficiency and handling requirements. In a preferred embodiment, nitrocellulose having a thickness of 0.5 mils to 2.0 mils for tissue sections 5-6 μm thick has been found to have the necessary qualities for use as the transfer membrane. Furthermore, pore size, surface porosity and surface texture of the transfer membrane are key parameters for providing acceptable cell morphology and spatial resolution of target signals. In this regard, the surface texture of the transfer membrane should preferably be flat, the pores should

be round having an actual measured diameter of between 0.22 and 2.0 μm and the surface porosity should be consistent with the flat structure, approximately 30%. It will be appreciated that an actual pore diameter measurement is a different measure than conventional "exclusionary" pore size measurements which are used elsewhere herein and in the membrane art.

The underlying support layer provides support, protection and rigidity to the membrane layer and associated specimen. Although the support layer can be nonporous, a porous layer is generally preferred having a porosity which facilitates the movement of water from the specimen into the transfer membrane by capillarity action, as well as providing a filtration format for decreasing reagent turnover times during the practice of the method. It will be appreciated that during practice of cytocoherent transfer of the present invention, cell solutes (including target solutes) will have been transferred to and retained in the transfer membrane. Three preferred embodiments of the present invention including a specimen slide, a tissue disc, and a specimen cassette are discussed below. It will be appreciated, however, that other configurations can be utilized without departing from the spirit and scope of the invention.

(1) Specimen Slide Device

Turning to Figure 10(A), a specimen slide device 10 is shown for preparing and observing a cytocoherent tissue specimen (not shown) through a microscope (not shown). The slide device 10 is comprised of a translucent support member 12 and a microporous transfer membrane 14 selected in accordance with the present invention, in which the support member 12 is a conventional microscope slide of the proper dimensions to enable it to be secured to a conventional microscope stage. The support member 14 satisfies the requirements of providing rigidity and support to the membrane 14, bonding to the membrane 14 in a continuous manner, maintaining optical clarity, and being resistant to chemicals used in cytochemistry. In this regard, the support member 12 can be glass or a suitable optical plastic providing the necessary optical characteristics. Silanized glass slides, such as *e.g.* Fisher Scientific SuperFrost Plus, are particularly suitable support members if the transfer membrane is to be spin cast thereon, as will be described in more detail below. The specimen slide device 10 will be positioned on the microscope stage by means of a conventional "slide holder" apparatus.

The transfer membrane 14 is secured to a top surface of the support member 12 by any suitable histology mounting attachment method, including but not limited to

casting, coating, the use of adhesives, polymer bonding, and welding before the assay process is performed, as discussed in particular detail in the Specific Examples below. The area size and shape of the transfer membrane 14 is shown here as being rectangular and covering approximately one-half of the surface of the support member 12, so as to accommodate a single tissue sample on the transfer membrane 14. The area size and shape are, however, generally not critical and can even accommodate more than one tissue sample.

Figure 10(B) shows a conventional coverslip 16 for a microscope slide completely covered with a layer of a transfer membrane 18 for the practice of the method of the present invention. The coverslip 16 provides the necessary support and ridgity to the transfer membrane 18. In this embodiment, the coverslip 16 is placed on a microscope slide, such as support member 12, such that the transfer membrane 18 and associated tissue sample are positioned between the slide and the coverslip 16, as is well understood in the art. The microscope slide can then be secured to a microscope stage by an appropriate slide holding apparatus for microscopic observation.

(2) Tissue Disc and Disc Holder

Turning now to Figure 11, a tissue disc 20 is shown including a disc-shaped support member 22 for supporting a plurality of circumferentially placed tissue sites 24. In the example as shown, the tissue disc 20 includes fourteen tissue sites 24, however, more or less tissue sites can be incorporated as desired. The tissue disc 20 is thus adapted to hold a numbered set of tissue or cell specimens, such as a pathologically coherent set, for cytochemical assay. What is meant by a pathologically coherent set is a plurality of samples organized, *e.g.* from one patient, from one tissue, or for one type of assay. Each of the tissue sites 24 includes a transfer membrane 26 holding a tissue sample 28 (see Figure 13) and the corresponding portion of the support member 22 thereunder. The support member 22 can be either a porous or non-porous, non-binding substratum for providing strength, durability and rigidity, and further to prevent creep of the transfer membranes. Specific examples of materials suitable for the support member 22 include glass, plastic, microporous thin films, and combinations and laminates thereof. In this regard, the member 22 has substantially the same properties as the support member 12 above. In a preferred embodiment, the support member 22 is a porous, non-binding cellulose acetate polymer, for example, cellulose triacetate, having a pore size larger than that of the transfer membrane 26, typically on the order of 0.45 microns. The pores in the member 22 facilitate capillary movement of water from the tissue samples 28 to the transfer membrane 26 during

cytocoherent transfer, as well as providing filtration for decreasing reagent turnover times. In certain applications, the member 22 can be a non-porous member of a semi-rigid material which is optically transparent and resistant to scratching, such as acrylic, MYLAR® or polycarbonate. A preferred thickness of the member 22 is between 0.002 and 0.01 inches. In one embodiment, the support member 22 is cut out as a circle and a binding polymer is applied to a surface of the support member 22. A patterned overlay, consisting of glass or plastic, is affixed to the support member 22 such that the binding polymer is exposed at open areas of the overlay. In another embodiment, circular strips are affixed and separated into discrete windows, preferably by sonication as generally described in U.S. Patent No. 5,075,077. Preferably, the affixation between the transfer membrane and the support member 22 is by direct casting or the use of adhesive material as described in the Specific Examples. However, the transfer membrane can be affixed to the member 22 by any of the suitable methods described herein. A bar code may also be incorporated on the tissue disc 20 for automated specimen code-recognition.

It will also be appreciated that each tissue site 24 could comprise a transfer membrane and a separate translucent underlying support member. In this manner, the support member 22 could be a non-translucent material, with cut-out sections at the tissue sites 24. Separate translucent support layers and transfer membrane would fill the cut-out sections. In yet another embodiment, the transfer membrane may be simply stretched across aperatures or windows representing the tissue site 24 formed in the member 22 without any underlying support member at the tissue sites 24. Certain problems, such as *e.g.* membrane creep, can, however, be encountered in the absence of an underlying support layer.

The support member 22 includes a centrally located hole 28 in order to enable the tissue disc 20 to be rotatably mounted to a tissue disc holder discussed below. Furthermore, the tissue disc 20 includes a cutout section 30 at its outer periphery in order to provide orientation of the tissue disc 20 relative to the first tissue site. In addition, a series of holes 32 are circumferentially provided such that the tissue disc 20 can be received by complementary projections on the disc holder, and thereby cooperate with the holder to precisely position the disc 20 in a predetermined position in the optical path of the microscope to provide direct and equal optical access to each tissue sample. Such positioning is preferred in order to minimize variations in the positioning of the tissue disc 20 relative to the light source of the microscope and the attendant position-induced variations in the readings in the test results from disc to

disc. The tissue disc 20 is positioned and locked in each of three predetermined dimensions: radially, circumferentially and vertically, as provided by the cutout section 28 and the holes 32.

Turning to Figure 12, a tissue disc holder 36 is shown according to a preferred embodiment of the present invention. The tissue disc holder 36 includes a support portion 38 which has right angle edges in order to be aligned and clamped to the microscope stage by a traditional microscope slide holding apparatus. A substantially circular platform portion 40 of the tissue disc holder 36 is provided in order to accept the tissue disc 20. In a preferred embodiment, the thickness of the support portion 38 is approximately the thickness of a conventional microscope slide, and the thickness of the platform portion 40 is of a thickness such that the combination of the platform portion 40 and the tissue disc 20 is substantially the same as the thickness of the support portion 38.

The platform portion 40 includes a cutout section 42 which will be aligned with the desirable tissue site to be viewed. Additionally, the platform portion 40 includes a centrally located center post projection 44 adaptable to be inserted within the center hole 28 of the tissue disc 20. Furthermore, a positioning ring 46 recessed within a positioning groove 48 is included to match up with associated structure (not shown) on the back surface of the tissue disc 20 in order to enable the tissue disc 20 to effectively rotate on the platform portion 40. A set of interlock projection pins 50 extend from the ring 46, and are provided to match up to the projection holes 32 on the tissue disc 20, as described above. In this manner, the tissue disc 20 can be manually rotated on the platform portion 40 in an effective manner so as to enable desirable tissue sites 24 to be aligned with the viewing window 42.

Turning to Figure 13, the tissue disc 20 is shown positioned within a tissue disc container 54. In this Figure, the tissue samples 28 are shown affixed to the tissue sites 24. Preferably, the container 54 has dimensions adequate to hold the tissue disc 20 in order to protect the tissue disc 20 during filing and storage. It is preferable that the container 54 be molded from a suitable plastic and the tissue disc 20 be sealed within a pouch 56 of metalized (*e.g.* aluminized) polyester such as MYLAR®. Furthermore, a lid 58 enables the pouch 56 including the tissue disc 20 to be inserted and removed within the container 54. For long term storage, the pouch 56 can be heat sealed under nitrogen or other inert gases. It may also be desirable to incorporate a bar code (not

shown) on the container 54 in order to provide information on the specimens enclosed.

(3) Specimen Cassette

In a specimen cassette embodiment, as depicted in Figure 5, a continuous strip of support material 60, such as polyester, is held within a cassette device 62 comprised of an unused cassette 64 and a used cassette 66. The continuous strip 60 includes a support layer and a number of tissue sites (not shown) comprising a transfer membrane and associated samples, as discussed above, in which the tissue sites are spaced apart. The continuous strip 60 is fed from the unused cassette 62 to the used cassette 64 in a conventional matter. The unused cassette 62 and the used cassette 64 are positionable in association with a light microscope such that the strip 60 extends therebetween carrying the tissue samples in the optical path of the microscope. In this manner, tissue samples in the unused cassette 62 can be continuously viewed as they are being drawn into the used cassette 62. Cassette devices of this type are generally known in the art, but are not applicable for the method of the present invention. For example, cassettes such as those described in U.S. Patent Nos. 3,526,480 and 5,077,010, are devised for the assay of fluids, but are not suitable for cytocoherent transfer. Likewise, because these devices are not designed for cytochemistry applications, they are not adapted for use in conjunction with conventional light microscopes.

C. SPECIFIC EXAMPLES

SPECIFIC EXAMPLE 1. Cytologically Coherent Transfer of Proteins to Nitrocellulose Membranes

a) Preparation of microporous membranes

Stock nitrocellulose transfer membranes used in the Specific Examples were obtained from Schleicher and Schuell (Keene, NH). Triton-free membranes with pore sizes of 0.05μm, 0.1μm, 0.2μm and 0.45μm (100-180μm thick) were impregnated (during manufacturing) with a proprietary non-cytotoxic wetting agent. Polyvinylidine difluoride (PVDF)-based hydrophilic Durapore membranes of 0.45μm pore size were obtained from Millipore Corp. (Bedford, MA).

Membranes were cut to the desired size (normally 1 X 1.25 in.) and affixed to plastic microscope slides by "spot-welding" the corners or an edge with a hot needle. Care was taken to avoid hand contact with working membrane surfaces.

b) Selection of membrane composition

Materials fabricated without fibrous interstices commonly used for nucleic acid and protein transfer from chromatographic gels, including nylons (*e.g.* Nytran® from

Schleicher & Schuell), PVDF-based membranes (*e.g.* Immobilon from Millipore, Inc.), and nitrocellulose all exhibit qualities important for preparing specimens for cytochemical assay using the transfer method of the invention. These qualities include resistance to reagents used in histology, optical clarity in histological mounting media, high capacity for binding cell RNA and soluble protein and blocking against non-specific retention of probes and signal development reagents. Performance differences among the materials (*e.g.* in wettability, ease of blocking, and resistance to alcoholic, acetone or formaldehyde fixatives), may require the tailoring of specific methods to suit a given material, which can be determined by those of ordinary skill in the art without undue experimentation.

Nitrocellulose was the preferred material because the optical clarity of the material was consistently superior to nylon or PVDF-based materials in the most commonly used xylene-based tissue mounting media and in immersion oil matched to the refractive index of the material. For example, N20/w was 1.50 for nitrocellulose as compared to 1.42 for PVDF (Immobilon and Duropore). Nitrocellulose also did not require prewetting for transfer whereas PVDF based-materials required wetting with alcohol or detergent prior to transfer. More rigorous blocking strategies are also required when alcohol-wetted membranes, as opposed to dry nitrocellulose are used. Figure 1 shows the appearance of membranes and tissue specimens thaw-mounted in accordance with the principles of the invention on nitrocellulose of 0.05μm pore size, after air drying and aqueous fixation (Figure 1A), after immunocytochemical staining (Figure 1B), and after counterstaining tissue with nuclear fast red and transparentizing the membrane in xylene and covership mounting using Permount (Figure 1C).

c) Tissue sample preparation

Breast cancer tissues were obtained from the Pathology Laboratory of William Beaumont Hospital, Royal Oak, MI. Tumor fragments were quick frozen in liquid nitrogen and stored at -70°C in evacuated air-tight plastic bags.

Tissue was sectioned using a Reichert-Jung cryostat at -20°C. Section thickness was nominally 5μm. Tissue sections were mounted onto slide-affixed membranes the same techniques comentionally used to mount frozen sections onto standard glass slides. A frozen thin section of tissue, which remains on the cutting surface of the cryostat knife after cutting, is brought into close proximity with the membrane surface by lowering the slide to the section. Paraformaldehyde (EM grade, Electron Microscopy Sciences, Fort Washington, PA), made 2% in phosphate buffered saline

(PBS, pH 7.4 containing 5mM $MgCl_2$) and containing 0.1% Tween-20 from Sigma Chemical, (St. Louis, MO) was used to fix sectioned tissue.

d) Freeze-thaw mounting tissue sections onto membrane to establish coextensive contact

Frozen thin-sections of breast tumor tissue were electrostatically attracted to dry nitrocellulose membranes affixed to plastic slides as described above and affixed evenly and adhered tightly to its surface at -20°C without physical encouragement. After adhering to the membrane, tissue was thawed and air-dried at room temperature.

Once sections were thawed and dried onto the surface of nitrocellulose membranes, tissue could not be removed intact. Even vacuum or pressure washing in "dot blot" manifolds, or washing under high stringency conditions used for *in situ* hybridization (continuous agitation in hypertonic saline containing 0.5% SDS at 80°C for 2 hrs.) did not dislodge tissue sections once dried on nitrocellulose. In contrast, nonporous glass or plastic substrates for tissue require special pre-coating with either poly L-lysine or 3-aminopropyltriethoxysilane or comparable materials for tissue to affix well. The coextensiveness of the contact between thin tissue sections and membrane was evaluated by means of scanning electron microscopy. The results of these observations, performed at 1000-3000X, demonstrated uniform coextensive contact between nitrocellulose and tissue when tissue was placed on dry membrane. The contact was not coextensive when tissue was placed on nitrocellulose prewet with aqueous buffer.

e) Fixation of tissue

The chemical sensitivity of nitrocellulose to alcohols and acetone suggested the use of an aqueous fixative. Paraformaldehyde was the aqueous fixative of choice for immunocytochemistry. See Drake, W.P. et al., *Cancer Res.* 32:1042-1044 (1972); Robinson, G., *Immunocytochemistry* in Theory and Practice of Histological Technique 406-427, eds. J.D. Bancraft and A. Stevens (1982). Presumably because the lipid-rich tissue was so tightly affixed to the hydrophobic membrane, addition of a wetting agent (0.1%m Tween-20) to the fixative was needed for rapid and uniform penetration.

Paraformaldehyde (EM grade, Electron Microscopy Sciences, Fort Washington, PA), made 2-3% in phosphae buffered saline (PBS, pH 7.4 containing 5mM $MgCl_2$) and containg 0.1% Tween-20 from Sigma Chemical Co., (St. Louis, MO) performed optimally. Membranes and affixed tissue were thoroughly wet in less than 1 min., and after 30 min. at 4°C, tissue was fixed to give excellent preservation of cell detail for morphometric or diagnostic purposes as shown in Figures 2 and 3. The Tween-20

also acted as a blocking agent for subsequent immunocytochemistry steps described below.

f) **Immunocytochemical assay methods**

A mouse monoclonal antibody recognizing a carbohydrate epitope in the extracellular domain of the transmembrane Her-2 receptor molecule (mAb-1) was obtained from Triton BioScience, Inc. (Alameda, CA). A polyclonal rabbit antibody (Ab-4) prepared against a synthetic peptide representing amino acid residues 1005-1016 of the epidermal growth factor receptor (EGFR) was obtained from Oncogene Science, Inc. (Manhasset, NY). Ab-4 recognizes an epitope in the cytoplasmic domain of EGFR. (See Gullick, W.J. et al., *Proc. R. Soc. London* 226:127-134 (1985). A mouse monoclonal antibody directed to an epitope of epithelial membrane antigen (EMA) (Sloan, J.P. et al., *Cancer* 47:1145-1149 (1983)) was obtained from Biomeda Inc. (Foster City, CA). Anti-A and Anti-B blood grouping antibodies, both murine monoclonal antibody blends, were obtained from Ortho Diagnostics (Raritan, NJ). All antibodies were diluted in TPBS containing 1.0% normal goat serum from Vector Laboratories, (Burlingame, CA).

Non-specific binding of primary antibody was blocked by pre-incubating tissue sections in TPBS containing 5.0% normal goat serum for 30 min. at room temperature. Tissue was then incubated with primary antibody at various dilutions for 30 min. to 3 hrs. at room temperature in a humidified atmosphere. Dilution and incubation times varied according to antibody type and empirically determined optimums. Following three 5-minute washes in TPBS, biotinylated secondary antibody (Vector Laboratories), diluted 1:500 in TPBS and 1.0% normal serum was then incubated with tissue for 30 min. at room temperature. Following three additional 5 minute washes and a second 30 min.-blocking in TPBS (without serum), color was developed using the Vectastain ABC-AP (alkaline phosphatase) kit (Vector Laboratories, AK5000), diluted 1:2 in TPBS pH 7.5 and subsequent incubation in an Alkaline Phosphatase substrate Kit (Vector Laboratories) prepared to manufacturer specifications, containing Levamisole (0.25mg/ml to block endogenous AP activity). Substrate solutions were filtered through 0.45μm cellulose acetate filters before use. Color was devoloped in the dark for 10-30 minutes. The length of color development time was determined by the emergence of non-specific staining on replicate control samples. Color development was ceased by washing sections in distilled water. After immunostaining, sections were counterstained, dehydrated and cleared for microscopic evaluation.

g) **Staining of tissue**

Histological counterstains were used to assist in the identification of morphological indentifiers and to enhance the contrast of immunocytochemical staining on freeze transfer specimens. The choice of counterstain was determined based on the ability to destain the membrane, and leave specific signal unobscured. Aqueous hematoxylin (Figures 2,7) and nuclear fast red (Figures 3, 4) were used routinely as nuclear counterstains. Eosin (prepared in isopropanol (50% v/v) was used as a cytoplasmic counterstain on nitrocellulose affixed specimens (Figures 2, 7). Destaining nitocellulose was readily accomplished by two 1-2 min. rinses in distilled water. After destaining and dehydration (by means of either air-drying or through an accending series of isopropanol concentrations) membranes were made optically transparent in oils, xylenes or histological mounting media. Permanent sections were made by coverslipping the specimens using Permount.

h) **Resolution of morphological markers in specimens**

Cell and tissue detail of frozen breast specimens fixed and stained as described above on nitrocellulose membranes (0.05µm pore size) was clearly discernable at 100-500X magnification. We could resolve without difficulty all the distinguishing epithelial and stromal elements of breast tissue generally used in diagnosis and prognosis. The functional cellular elements of secretory ducts, including lobules, extra- and intra-lobular ducts and myoepithelium were clearly recognizable, as were different morphological types of benign and malignant tumors (Figure 2). Figure 2A illustrates a mitotic cell, in a collection demonstrating nuclear atypia, which is clearly discernable on the .05µm pore size nitrocellulose-affixed 5µm frozen tissue sections.

i) **Effect of membrane pore size on cytomorphology**

Thin sections of one breast carcinoma were thaw-mounted on dry nitrocellulose membranes of 0.05µm and 0.45µm pore size, air-dried, fixed with paraformaldehyde, and stained with hematoxylin and eosin. The results are illustrated in Figure 7, showing the superior morphologic resolution (detail) using 0.05µm pore size (Figure 7A) as compared to 0.45µm pore size (Figure 7B).

j) **Planar definition of immunochemical signals on nitrocellulose membranes**

Commercially available alkaline phosphatase-based reagents (Vector Laboratories) were used as described above to colorimetrically localize Her-2 and milk fat globule (EMA) glycoproteins in breast tissue specimens thaw-mounted onto nitrocellulose membranes (0.05µm pore size) as described above. The well-characterized plasma membrane and cytoplasmic location of these markers. Sloan,

J.P. et al., *Cancer* 47:1145-1149 (1983); van de Vijver, M.J. et al., *N. Engl. J. Med.* 319:1239-1245 (1988) provided a reliable reference standard to evaluate the effect of transfer on spatial resolution of soluble receptor protein in cells. Immunochemistry was conducted as described in the methods section. Tissue was counterstained with nuclear fast red as described above.

As shown in Figure 3, EMA was characteristically localized in the apical cytoplasm of secretory cells, in the extracellular secretional lumen (Figure 3A), and in the plasma membrane of non-secretory breast epithelium (Figure 3B).

The Her-2 receptor was also specifically localized in the plasma membrane of breast epithelial cells (Figures 3C and 3D), which is the characteristic location for that receptor. van de Vijver, M.J. et al., *N. Engl. J. Med.* 319:1239-1245 (1988). Figures 3 and 4, described below, also illustrate the optical quality of immunocytochemical specimens on nitrocellulose membranes and the negative backgrounds on breast stroma and transfer membrane.

k) Effect of membrane wetness and pore size on signal resolution and intensity

The immunochemical Her-2 signal was resolved cytologically only when tissue sections were mounted on dry nitrocellulose membrane and post-fixed. The signal was diffused over the entire subcellular surface of the transfer membrane when tissue was mounted on membrane pre-wet with either PBS, TPBS or paraformaldehyde (2%) in TPBS. Figure 4 compares the effect of thaw mounting tissue on dry (A,C) and prewet (B,D) membrane.

Cytologic resolution of Her-2 signal was also affected by the pore size of the transfer membrane. The signal was most coherent on membranes with 0.05μm pores (Figures 4A, B), and most diffuse on membranes with 0.45μm pores (Figures 4C, D). Spatial resolution of signal was inversely related to pore size between 0.05μm and 0.45μm, independent of the membrane material used (Immobilon P (PVDF) or nitrocellulose).

In contrast to signal resolution, signal intensity was directly related to membrane pore size between 0.05μm and 0.45μm. Results of antibody dilution experiments (Figure 5D) indicated that Her-2 signal was 3-6 times more intense when a given tissue was thaw-mounted on membranes with 0.45μm pores than with 0.05μm pores. These cytologic data were in agreement with densitometry readings on segments of the unmagnified tissue sections (c.f. Figure 4 insets),

Reactive cells could be readily enumerated on membranes with 0.45μm pores (Figure 5D), but fine structure cell morphology was too distorted on these larger pore

membranes for diagnostic analysis (Figure 4D). The diffusional effect of pre-wetting membrane was independent of pore size (Figure 4).

l) **Sensitivity of freeze-thaw transfer method**

In comparative tests of 11 breast tissues, the method of the invention utilizing 0.05µm pore size nitrocellulose with freeze-thawing of the sample as described above was 25-40 times more sensitive than conventional thaw-mounting on glass, based on signal:noise at progressive antibody dilutions. Of the 11 tumors tested, 9 were reactive with Her-2 antibodies (mAb-1), and 3 were reactive with EGFR antibodies (Ab-4).

Typical assay results are shown in Figure 5. Glass slides were silanized (Rentrop, M. et al., *Histochem. J.* 18:271-276 (1986)) before mounting tissue. All transfer membranes were used dry; and all samples were post-fixed with paraformaldehyde (2%) in TPBS. After immunochemistry reactions were complete, tissue was counterstained with nuclear fast red. Cells, identified by nuclear and unit membrane morphology, were counted at 400X. Reactive cells were those with alkaline phosphatase-dependent plasma membrane staining; cytoplasmic staining was discounted. Percent reactive cells was determined as the average of three high-power fields in duplicate serial tissue sections. Test conditions are indicated on the graphs: glass-tissue thaw-mounted on glass microscope slides; 0.05µ n/c - tissue thaw-mounted on nitrocellulose transfer membranes with 0.05µm pore size; PVDF - tissue thaw-mounted on hydrophilic PVDF (Durapore) membranes; anti-EGFR-Ab-4, used as primary antibody rather than anti-Her-2 (mAb-1) on 0.05µm n/c. The four neoplasms represented in Figure 5A-D qualitatively "over-expressed" Her-2 (relative to normal cells) and contained amplified Her-2 sequences in DNA (data not shown). In one sample of malignant cells (Figure 5A), Her-2 reactivity diluted to extinction at 1:20,000 on nitrocellulose, and at 1:500 on glass slides. In a sample of benign intraductal cancer cells (Figure 5B) reactivity diluted to extinction at 1:2000 on glass slides, and at l:50,000 on membrane. For comparison of assay sensitivity between freeze-thaw transfer and paraffin section, the highest dilution of mAb1 that detected Her-2 protein on paraffin section of either tissue (5A or B) was 1:100. Figure 5D illustrates a breast tumor in which no Her-2 signal was detected in cells mounted on glass slides whereas on nitrocellulose virtually every cell was reactive, at 10-20 fold higher antibody dilutions.

The enhanced sensitivity of freeze transfer was also evident by the improved duplication (quantitation) of data at the cell level, a finding which indirectly indicated that thaw-mounting thin tissue sections on microporous membranes controlled for the loss of soluble protein targets. However, control of target loss on nitrocellulose

membranes was still related to pore size (Figures 5C and 5D) and the need to balance signal loss against signal resolution. Figures 5B and C show that assays of tissue freeze-thawed onto hydrophilic PVDF membranes (0.45μm pore size) which bind RNA and protein poorly (<1μg/cm^2) were nearly equal in sensitivity to thaw mounting on glass, 25-50 times less sensitive than thaw-mounting on nitrocellulose of the same 0.45μm pore size. (Figure 5C).

The closely related EGF receptor could be readily distinguished from Her-2 receptor in breast carcinoma cells (Figures 5B and C; Figure 6), which demonstrates the specificity of the immunocytochemical reaction and low non-specific backgrounds in freeze transfer assay format. The two tumors shown in Figures 5B and C were unreactive with anti-EGFR antibodies when assayed on either glass or nitrocellulose membranes (0.05μm or 0.45μm pore size). The specificity of immunocytochemical reactions performed in freeze transfer assay format was also demonstrated in patients differentiated into A and B blood groups. In breast tumors from these patients, endothelial cells specifically were reactive with Type-A or Type-B antibodies; and epithelial (carcinoma) cells specifically were reactive with Her-2 or EGFR antibodies.

Figure 6 illustrates the low control antibody backgrounds in assays utilizing the method of the present invention. It also illustrates the enhanced signal obtained in assays performed on nitrocellulose-affixed tissue samples, compared to glass. Serial 5μ frozen sections of the same breast cancer tissue, were thaw-mounted on nitrocellulose (0.05μ pore size) membranes (Figures 6A and C) and silanized glass slides (Figure 6B). Tissues were reacted with Her-2 (A,B) and EGFR (C) antibodies (mAb-1 and Ab-4). The mAb-1 dilution in A was 1:2000; in B, was 1:200. The Ab-4 dilution in C was 1:200. Secondary antibody was used at 1:500 in all. Tissues were counterstained with nuclear fast red (X400).

SPECIFIC EXAMPLE 2. Cytologically Coherent Transfer of Her-2 mRNA to Nitrocellulose Membranes

Tissues were prepared by the freeze transfer method of Example 1 (a-f). After fixation in paraformaldehyde solution, samples were held under a hand-held ultraviolet light source for 30 sec. After fixation, tissue sections were soaked (blocked) in 4X SSC containing 0.1% Tween-20 for 10 min. at room temperature. Blocking was followed by 1 hour incubation at room temperature with 50-100μl prehybridization buffer (50% formamide, 4XSSC, 1X Denharts, 250μg/ml denatured salmon sperm DNA, 400ug/ml yeast tRNA and 0.1%SDS, pH 7.2).

A cDNA probe, biotinylated by nick translation, was added to prehybridization buffer (50ng/ml). Incubation was at 37° C for 4-16 hours. High stringency washing was accomplished by two 15 minute incubations of tissue in 4X SSC at room temperature; followed by two 15 minute incubations in 0.2X SSC at room temperature. Blocking and color development were done as described in Specific Example 1(f).

Upon microscopic examination Her-2-specific mRNA was confined to the cytoplasm, specifically of epithelial cells, of benign and malignant breast cancer cells (Figure 9). The intensity of colorimetric signal indicated a 20-35 fold increase in sensitivity, compared to the samples affixed by conventional methods to gross microscope slides. Parallel quantitative experiments with radioactively labelled probes indicated that 70% or more of Her-2 mRNA was lost from cells freeze thaw-mounted on glass slides during the manipulations of *in situ* hybridization, and that RNA was retained on nitrocellulose membranes in a cytologically coherent distribution.

SPECIFIC EXAMPLE 3. Long-Term Specimen Storage

Thin cryostat sections of breast cancers prepared and fixed according to the freeze transfer method of Specific Example 1 were stored for 12 months at room temperature without loss of either resolution or intensity of Her-2 signal, determined by the method of Specific Example 1(f). Storage of the air-dried specimens was in an oxygen and light-deprived dessicated atmosphere. Fixation was found to be essential for long-term storage.

SPECIFIC EXAMPLE 4. Cytologic Preparation of Plant Cells on Microporous Nitrocellulose Membranes

Frozen sections of two types of herbacious specimens, *Zea mays* and *Cercium canadensis* were prepared as follows: within 30 minutes after collection, specimens were cut into small pieces approximately 1cm in length, secured to wooden tongue depressors using OCT (Miles Inc.) in longitudinal and cross-section orientations, then submerged in liquid nitrogen until completely frozen. Samples were transfered to plastic bags and stored at -70°C. Before sectioning, samples were allowed to warm to -20°C in the cryostat (30 min). Serial sections of 5, 10 and 20μm were made of each sample in each orientation. Sections were mounted onto silanized glass slides and dry nitrocellulose (0.05 and 0.45μm pore size) membranes as described in Specific Example 1.

Plant tissue sections affixed poorly to glass slides specially treated (silanized) to effect maximum adherance. Even after drying, sections did not affix well enough when placed in fixative and staining solutions to prevent loss and/or distortion of tissue. In

contrast, plant tissue sections affixed easily to nitrocellulose membranes and affixed tightly enough to be placed directly (without drying) into fixatives and staining solutions without loss or misalignment of tissue. This advantage of nitrocellulose transfer membranes was particularly noteworthy when preparing thick (greater than 10μm) plant tissue sections for cytochemical assay.

The paraformaldehyde fixative (3% in TPBS) used in Specific Example 1 preserved morphology of plant cells equally well. In addition to paraformaldehyde, other common plant fixatives (picric acid, glacial acetic acid (up to 5%), isopropyl alcohol (up to 95%)(see Johansen, D.A. in: Plant Microtechnique, McGraw-Hill (New York 1940), also worked successfully to preserve morphological detail of plant cells on nitrocellulose membranes for cytological assay. Aqueous counterstains including Acid fuchsin, Aniline Blue, Crystal violet, Hematoxylin and Safranin O all stained the appropriate anatomical structures in freeze-transferred plant cells, as did alcoholic Eosin Y and Fast green FCF (prepared in isopropyl alcohol 50% v/v) see Botanical Microtechnique and Cytochemistry G.P. Berlyn and J.P. Mikscher (eds.), The Iowa State University Press (Ames, Iowa 1976).

Microscopic examination of stained specimens at 100 and 400X showed that plant cell morphology was as well preserved on 0.05μm nitrocellulose transfer membranes as on glass. Vascular bundles containing xylem and phloem, dermal cells, cuticle and epidermal cells were clearly identified. Parenchyma plant cell morphology, like parenchyma animal cell morphology described previously, was compromised in preparations affixed to 0.45μm pore size nitrocellulose membranes. Ground tissue (collenchyma and sclerenchyma) cells were not unduly distorted on 0.45μm pore size freeze-transfers. However, the contact between the cut surface of cells and the underlying microporous transfer membrane was coextensive only in the case of 0.05μm pore size. The thickness of biological membranes comprising and delimiting plant parenchyma cell organelles was determined to be approximately 0.1μm in fixed and stained freeze-transfer preparations, which also indicates that the pore size requirements of the transfer membrane for cytocoherent transfer of macromolecules from plant cell samples is the same as for animal cell samples.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings and claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

SPECIFIC EXAMPLE 5. Lyophilization

Breast cancer tissue was cut into approximately 5mm by 1mm pieces, was quench frozen in chilled isopentane, cut into 6μm sections at -35°C, mounted onto nitrocellulose films cast on a glass slide and impregnated with SDS. The specimens were thaw-mounted in transit to a vacuum chamber in which the specimens were refrozen and dried to 1-2% moisture at -35°C under 10-3 mm Hg (final vacuum). The samples were stained with hematoxylin (0.4% aqueous) and eosin (0.5% in 95% isopropanol) and a photomicrograph taken to examine the results.

Ice-crystal artifact was not apparent at 400X (resolving power = 0.2μm), and tissue morphology was clear enough to classify cancers within existing systematics. Nucleoli and mitotic figures were easily discernable and an area of normal duct cells was also visible, demonstrating luminal secretion normally lost from glass-affixed specimens. The photomicrograph showed that lyophilization on small pore surface cast thin films did not compromise the method. A photomicrograph (400X) of the same sample after three freeze-thaws showed the deleterious effect of uncontrolled ice recrystallization due to multiple freeze-thaws of tissue on cell and tissue morphology after lyophilization.

SPECIFIC EXAMPLE 6. Membrane Formulation

A preferred formulation of a transfer membrane or film for casting in accordance with the present invention is as follows:

Solution A

In a 250 ml Erlenmeyer Flask containing a magnetic stir bar, placed atop a magnetic stir plate, 20.0g nitrocellulose (Hercules, viscosity RS-23 cps) was added to 20.0g n-butyl alcohol and 2.0g distilled water. 25.0g acetone was added while stirring. Stirring using high speed continued until the nitrocellulose was completely dissolved and a smooth mixture was formed.

Solution B

In a separate flask, 20.0g nitrocellulose (Hercules, viscosity RS-8 sec.) was added to 20.0g butanol. 35g acetone was added while stirring using high speed until the nitrocellulose was completely dissolved and a smooth mixture was formed.

Solution C

In a separate flask, 60.0g of Solution A and 60.0g of Solution B were combined and stirred. 8.2g butanol, 3.28g distilled water and 1.64g isopropyl alcohol was added and stirred until completely mixed (approximately 30 minutes).

Particulates were removed from the resulting blend of materials either by filtration or centrifugation. Care was taken to limit exposure to the air and loss of volatile solvent from the solution.

A surfactant, such as SDS or DOC, is preferrably added to the polymer solution, e.g. as a component of the water fraction. The surfactant can also be mixed in powder as well as a liquid form. The amount of surfactant can be varied to optimize desired mobilization effects without sacrificing cell morphology.

SPECIFIC EXAMPLE 7. Spin-Coating

Transfer membrane was cast on the support member by spin coating. A piece of support material was secured to the flat upper surface of a centrifuge rotor. 1.0 ml of the polymer Solution C, both with and without SDS, as described above in Specific Example 6, was pipetted onto the surface of a slide having a surface measuring 1.0 inch by 3.0 inches. The application of the solution was made while the slide was in a stationary position. Immediately after application of the polymer solution, centrifugal force was applied to the slide by a centrifuge device in a manner well known to those skilled in the art. During rotation, the polymer solution leveled out in the centripetal field. After the spin time was complete, the slide coated with the wet film was removed from the centrifuge device and treated to promote phase inversion and membrane formation.

In the phase inversion process, the resulting film was exposed to air or dipped into a second solvent extracting agent, such as water, to extract the solvent. This extraction of the solvent results in a phase separation of the polymer film attendent with polymer precipitation and microporous film formation. Factors such as viscosity grade, co-solvent/solvent ratios, coating procedures, wet film thickness and conditions for phase inversion are important to the development of the film are known to those skilled in the art. **SPECIFIC EXAMPLE 8. Film Applicator**

In another method of casting, an adjustable micrometer film applicator was used. In this application, the adjusted applicator was positioned above the surface to be coated, ensuring that this surface was firmly held on a uniform and flat base. An adequate pool of polymer solution, such as Solution C, was then placed near a blade of the applicator. By adjusting the sides of the applicator and pulling it along the surface through the pool of material at an even and uninterrupted rate to the end of the member, uniform coating was obtained. The wet film thickness was gauged by a micrometer adjustment on the applicator and the viscosity of the polymer solution. Phase inversion was accomplished as described in Specific Example 7 above.

SPECIFIC EXAMPLE 9. Adhesive Binding

The transfer membrane was also affixed to the support member with an adhesive. Certain pressure sensitive adhesives, such as ARcare 7843 and ARcare 7840, both commercially available, are clear, pressure-sensitive adhesives with a peel-off backing material. These adhesives are particularly suitable for bonding, laminating, and assembly of *in vitro* immunodiagnostic and related assay products, and are well suited as a component material for fabrication of membrane-based solid phase immunoassay systems.

An exposed adhesive surface was applied using a roller to one surface of the transfer membrane. An opposite adhesive surface was exposed by peeling off the remaining backing sheet and the adhesive backed film was applied to the member using a roller. When transfer membranes are affixed using these methods, mineral oils rather than xylenes are used as a choice of clearing agent.

SPECIFIC EXAMPLE 10. Welding

Another method of affixing the transfer membrane to a support is heat bonding by the use of a heating iron or sonication. In this method, the transfer membrane is placed atop a support layer, and the smooth surface of a heating iron or sonicating device is pressed against the area to be bonded such that the resulting effect heats the area locally for controlled melting and welding of the support material to the transfer film.

SPECIFIC EXAMPLE 11. Polymer Bonding

Another method of affixing the transfer membrane to a support includes polymer bonding. A polymer solution such as Solution C was used to form the transfer membrane. Bonding polymers, both with and without pore forming properties, were used to bond the transfer membrane material the support material. One suitable non-pore forming bonding polymer used was RS 30-35 cps 30g dissolved in 25g acetone.

Depending on its pore characteristics, the bonding polymer may serve to provide a porous intermediate layer between the support and the transfer membrane or it may only serve as a chemical "glue." This method works particularly well when a thin layer of a bonding polymer is cast onto a cellulose acetate sheet material and a piece of microporous nitrocellulose is joined by using a roller and even pressure.

All publications cited herein are incorporated by reference.

IN THE CLAIMS:

1. A method of preparing a cytocoherent specimen of a biological sample from which a target cell solute is transferred and retained in a microporous transfer membrane in accurate planar definition relative to cell structures in the biological sample comprising the steps of:

a) providing a biological sample;

b) providing a microporous transfer membrane, the step of providing the transfer membrane further comprising the step of selecting a membrane with the following characteristics:

1) pore size no greater than approximately 0.1 μm;

2) porosity which does not disrupt substantial molecular continuity between the sample and microporous membrane when they are contacted; and

3) a composition with binding affinity for and having sufficient binding capacity for the soluble contents of the specimen and which is resistant to the agents used for target solute transfer;

c) providing substantially molecularly continuous contact between the sample and the transfer membrane under conditions favorable for target solute transfer and unfavorable for lateral diffusion of solute on the transfer membrane; and

d) lyophilization of the sample onto the membrane.

2. The method of Claim 1, wherein the transfer membrane is impregnated with a surfactant.

3. The method of Claim 1, wherein the sample is frozen and the method further comprises the step of thawing of the sample on the transfer membrane.

4. A method of preparing a cytocoherent specimen of a biological sample from which a target cell solute is transferred and retained in a microporous transfer membrane in accurate planar definition relative to cell structures in the biological sample comprising the steps of:

a) providing a biological sample;

b) providing a microporous transfer membrane, the step of providing the transfer membrane further comprising the step of selecting a membrane with the following characteristics:

1) pore size no greater than approximately 0.1 μm;

2) porosity which does not disrupt substantial molecular continuity between the sample and microporous membrane when they are contacted; and

3) a composition with binding affinity for and having sufficient binding capacity for the soluble contents of the specimen and which is resistant to the agents used for target solute transfer;

c) impregnating the transfer membrane with a surfactant; and

d) providing substantially molecularly continuous contact between the sample and the transfer membrane under conditions favorable for target solute transfer and unfavorable for lateral diffusion of solute on the transfer membrane.

5. The method of Claim 4, wherein the sample is frozen and the method further comprises the step of thawing of the sample on the transfer membrane.

6. A specimen device for cytocoherent transfer of a selected biological sample, the device comprising:

a support member, the support member including a portion translucent to light; and

at least one transfer membrane affixed to the support member, the transfer membrane being a porous membrane in which the pore size is no greater than the average thickness of the biological membrane domain of the cell structure with which the cell target solute associates and which does not disrupt substantial and molecular continuity between the sample and the transfer membrane when they are contacted, in which the porosity does not disrupt substantial molecular continuity between the sample and the transfer membrane when they are contacted, and having a composition with binding affinity which has sufficient binding capacity for the soluble contents of the sample selected for transfer and which is resistant to the agents used in the transfer method.

7. The specimen device according to Claim 6 wherein the support member is porous having an average pore size larger than the pore size of the transfer membrane.

8. The specimen device according to Claim 6 wherein the support member is a microscope slide comprised of a material selected from the group consisting of glass and optical quality plastic.

9. The specimen device according to Claim 6 wherein the support member is a microscope slide coverslip.

10. The specimen device according to Claim 6 wherein the support member comprises a tissue disc including a plurality of circumferentially located tissue sites, wherein each tissue site includes a transfer membrane.

11. The specimen device according to Claim 10 wherein the support member is comprised of a material selected from the group consisting of glass and an otpical plastic, and wherein the tissue sites are defined by an overlay layer positioned on the support member, the overlay layer including cut-out sections.

12. The specimen device according to Claim 10 wherein the support member comprises an acetate polymer.

13. The specimen device according to Claim 10 further comprising a tissue disc container, said tissue disc container including an inner pouch container for sealing the tissue disc therein and an outer container for accepting the pouch container, said outer container including a lid.

14. The specimen device according to Claim 6 wherein the support member comprises a continuous support strip wound within an unused cassette, wherein the continuous strip includes a plurality of tissue sites, each tissue site including a transfer membrane, the device being operable to be positioned on a microscope such that the continuous strip is extracted from the unused cassette and wound around a used cassette.

15. The specimen device according to Claim 6, wherein the transfer membrane is impregnated with a surfactant.

16. The specimen device according to Claim 6 wherein the transfer membrane is cast on the support member.

17. The specimen device according to Claim 6 wherein the transfer membrane is affixed to the support member by means of an adhesive.

18. The specimen device according to Claim 6 wherein the transfer membrane is welded to the support member.

19. The specimen device according to Claim 6 wherein the transfer membrane is comprised of a material selected from the group consisting of nitrocellulose, a nylon-based material and a PVDF-based material.

20. The specimen device according to Claim 6 wherein the transfer membrane has a pore size of no greater than about 0.1μm.

21. The specimen device according to Claim 6 wherein the transfer membrane comprises nitrocellulose cast on the support member, wherein the transfer membrane has an actual pore diameter in the range of approximately 0.22μm to approximately 2.0μm, a thickness of at least approximately 0.5 mil and a porosity of approximately 30%.

22. A specimen device holder for holding and positioning a tissue disc used in cytocoherent transfer of a selected biological sample, the tissue disc including a plurality of circumferentially located tissue sites, the holder comprising:

a base portion operable to be clamped to a microscope stage; and

a platform portion operable to accept the tissue disc, the platform portion including a centrally located projection adaptable to be inserted in a centrally located hole is the tissue disc and a plurality of circumferentially positioned projections engagable within circumferentially positioned holes in the tissue disc, wherein the tissue disc is rotatable relative to the platform.

23. The specimen device according to Claim 22 wherein the platform portion includes a ring portion positioned within a recess in the platform portion, the ring portion engagable within a groove in the tissue disc.